



## The response of stressed and unstressed cultivars of pearl millet to *in vitro* conditions appears similar

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### Publication History

Received: 15 June 2014

Accepted: 19 July 2014

Published: 1 August 2014

### Citation

Masoumeh Yaghoobi, Niloofar Hashempour, Mouza Al Shamisi, Aly ZE Abdelsalam, Rajya Lakshimi. The response of stressed and unstressed cultivars of pearl millet to *in vitro* conditions appears similar. *Discovery*, 2014, 22(73), 49-54

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### General Note

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### ABSTRACT

Pilot sea water farming with pearl millet [*Pennisetum glaucum* (L.) R.Br.] cultivars has been attempted. Petri plate screens and pot cultures have been used to screen for salt tolerant genotypes among four cultivars of pearl millet. Sea water dilutions 1/4<sup>th</sup>, and 1/8 with electrical conductivity of 6.8 dS/m and 13.6 dS/m were used to identify the tolerant and sensitive cultivars. Based upon the parameters- per cent seed germination, seedling growth, ratio of root length to shoot length, the cvs 19612 and IP 6106 are found to be the most tolerant and cvs IP 22269 and IP 6098 the least tolerant to sea water dilutions, reflecting genotypic differences among the four cultivars. The selected seedlings of cvs IP 6106 and IP 22269 and of after being stressed against 150mM NaCl solution for 1 week, were cultured in tissue culture media. The controls of both cultivars developed 4-6 multiple shoots in MS+3mg/L BAP medium in 4 weeks, while the salt stressed seedlings of the respective cultivars developed 2/3 shoots in 8 weeks. The leaf explants of controls

and of stressed seedlings callused in 2 weeks in MS+2.5mg/L 2,4-D media, the regeneration results are awaited. The response of stressed seedlings and their respective unstressed seedlings to *in vitro* conditions appears similar.

Keyword - micropropagation, NaCl, salt tolerance, sea waters, pearl millet cultivars.

## 1. INTRODUCTION

Environmental stresses such as high temperature, drought and salinity are the prime limiting factors that curtail agricultural productivity. Salinity is an abiotic stress that affects the development and physiology of plants thereby lowering the crop productivity. Less than a decade back it has been reported that one third of all irrigated lands in the world are affected by salinity and the problem is expected to increase in future [1]. Expansion of irrigation does not seem feasible in many countries in Asia, North Africa and Middle East, where the present study is being carried as most of the available water sources have already been utilized [2]. To improve the productivity of such saline soils there is an upsurge need for salt tolerant crops that suit better to the harsh environments. There is hope that these salt tolerant crops do not require as much water as the unstressed crops [3].

Cereal crops, an important source of human food and cattle feed next to legumes, are more tolerant to abiotic stresses than legumes. Among the cereals, pearl millet [*Pennisetum glaucum* (L.) R.Br.] reported to be more tolerant to saline soils [4], is widely cultivated for grain and fodder in Arid and semi-arid zones of South Asia and West Africa where the soils are prone to drought and salt stress. Wide genetic variability for salinity tolerance (7.5 – 8.25 dS/m) has been reported for different germplasm accessions, cultivars and hybrid parental lines in pearl millet grown under pot and field conditions [5]. Salt tolerance of pearl millet is genotype dependent [6] different varieties have different levels of salinity tolerance [7]. Different developmental stages of a plant tolerate salinity differently; the performance of seedlings under saline conditions is considered to represent the response of the adult plants to salinity [8]. Hence, the emergence and early seedling growth of four cultivars of pearl millet IP 19612, IP 6106, IP 22269 and IP 6098 are chosen for the present investigation. The chosen four cultivars could withstand salinity as high as 27dS/m, at seed germination and early growth and the varieties differed among themselves in their salt tolerance [9]. Pearl millet being an open pollinated crop, clonal propagation is mandatory to breed the selected salt tolerant genotypes. Although a body of literature is available on tissue culture and plant regeneration of almost all grasses, data on regeneration protocols for salt tolerant cultivars is lacking [10 & 11] which is the aim of the present study.

The objective of the present study was to screen the four resistant genotypes at seedling stage for salinity tolerance against  $\frac{1}{4}$  and  $\frac{1}{8}$  dilutions of sea water and micropropagate the selected, tolerant seedlings through tissue culture techniques.

## 2. MATERIALS AND METHODS

### A. Short term experiment

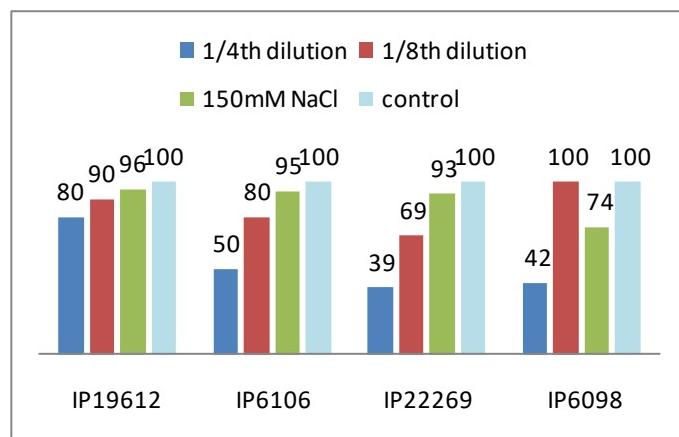
#### *Seed germination in sea water dilutions and NaCl solutions:*

The seeds of four cultivars IP 19612, IP 6106, IP 22269 and IP 6098 were obtained from International Centre for Biosaline

agriculture, Dubai, UAE. Sea water was collected from Juremiah beach residence, Dubai and diluted to  $\frac{1}{4}$  and  $\frac{1}{8}$  with distilled water. Complete sea waters or  $\frac{1}{2}$  dilutions of sea water were not tested as the seeds of these cultivars did not germinate in those solutions in our earlier trials. For each cultivar 90 seeds in three replicates were germinated on moist filter paper in small Petri plates containing distilled water, or  $\frac{1}{4}$  or  $\frac{1}{8}$  diluted sea water. Thirty seeds were incubated for germination in 150mM NaCl solutions. For micropropagation experiments, the seedlings were grown in 150mM NaCl solutions for a week in order to stress the seedlings against a definite amount of NaCl. The Petri plates were incubated at 25°C under 12hr light and 12hr dark photoperiod. The measurements of the root and of shoot were made with the help of thread and graph paper.

### B. Long term experiments:

About 25 seeds of each cultivar were sown in the field, irrigated with tap water were grown to maturity. The temperature was 30 -35 °C during day time and 25 -28°C in the night. Twenty five seeds, 5 per pot of each cultivar were irrigated with sea water dilutions were grown up to two months. About 250gms of autoclaved soil was taken in each pot, seeds were sown, were watered with 150 ml of diluted sea water each day. Pots were incubated at 25°C under photoperiod of 12hr light/12hr dark.



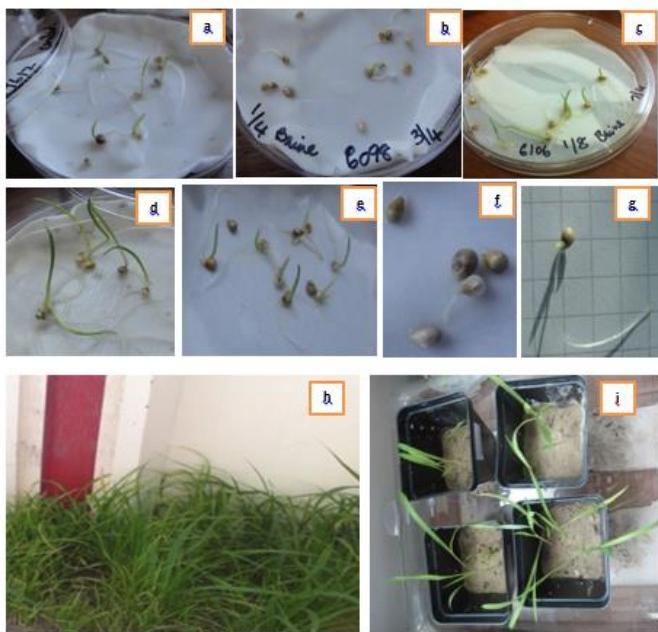
**Figure 1** Per cent seed germination of four cultivars of pearl millet in different salt solutions.

### C. Tissue culture media:

The control seedlings and the 2- week old salt stressed seedlings (1 week in  $\frac{1}{4}$  diluted sea waters and 1 week in 150mM NaCl solution) were surface sterilized with 0.01 % mercuric chloride for 15 mins and were washed with sterile water for 3-5 times. The surface sterilized seedlings are cultured in MS basal medium [12] supplemented with 3.0mg/L BAP, 3% sucrose and 0.8% agar (Hi media, Mumbai, India) media. The cultures were maintained at 24±2°C with a photoperiod of 16 hr light/dark. The single shoots are subcultured in MS+1.0mg/L

NAA medium. The plantlets were being acclimatized in autoclaved soil.

The leaves from plants stressed for 2 weeks are cultured in MS+ 2.5 mg/L 2,4-D medium. Usually, there are two leaves for the control seedlings, while the stressed seedlings had only one leaf, which are cut into 0.5cm long segments and cultured. The cultures were maintained in [dark at  $24\pm2^{\circ}\text{C}$ . The concentrations of the growth hormones are so chosen, which gave positive results with the explants of the same cultivars in the previous experiments [9]. Cultivars IP 6106, the salt tolerant one and IP 22269, the salt sensitive are chosen for the *in vitro* experiments.



**Figure 2** Seed germinations of 4 cultivars in salt water, sea water dilutions and in soil irrigated with salt waters.

Figure a: One day old seedlings of cv IP 19612 germinated in water.

Figure b: Four day old Seedlings of IP 6106 germinated in 1/4th diluted sea water.

Figure c: Four day old seedlings of cv IP 6098 in 1/8th diluted sea water.

Figures d-g : One week old seedlings.

Figure d: Control seedlings of cv IP 19612.

Figure e: Seeds of cv IP 19612 incubated in 1/4 dilution of sea water.

Figure f: Seedlings of cv IP 19612 in 1/8<sup>th</sup> diluted sea water.

Figure g: Long rooted seedling of cv IP 6098 incubated in 1/8<sup>th</sup> dilution of sea water.

Figure h: One month old control plants of four cultivars in field conditions.

Figure i: One month old seedling of four cultivars irrigated with 1/8<sup>th</sup> dilution of sea waters.

### 3. RESULTS

#### A. Seed germination of the four cultivars of pearl millet in the 1/4, and 1/8<sup>th</sup> dilutions of sea water (JBR beach) and 150mM NaCl solutions

The seed incubated in water, germinated in 1 day (Fig.2a), while those incubated in sea water dilutions germinated by 3rd or 4th

day. In the 1/4<sup>th</sup> dilution of sea waters, the germination was noticed only by the 4<sup>th</sup> day in all four cultivars (Fig. 2b). The cultivar IP19612 had the highest number 80% of seed germination while cv IP 22269 had the least number (38.4%) of seed germination (Figure 1). In the 1/8<sup>th</sup> dilution of sea waters, 100% of the seed germinated in the cv IP 6098, while only 69% germinated in cv IP 22269, (Figure 1). The seedlings were healthy among all four cultivars, though smaller in size (Fig. 2c). By one week, the growth was enhanced in the roots than in the shoots, the roots being much longer than of the shoots (Fig.2 g). In a few seedlings of cv IP 19612 salt deposition is noticed on surface that did not hamper its germination or further growth (Fig.2 f). In both dilutions of sea water cv IP 22269 had the least per cent of seed germination (Fig.1). The first leaf was still enclosed with in the coleoptile of the seedlings. The seeds incubated in 150mM NaCl germinated in 1 day, per cent of germination ranged between 74% to 95% among the four cultivars, the sensitive genotype being cv IP 6098 (Fig.1).

#### B. Comparison of root length to shoot length among four cultivars in different dilutions of sea water and 150mM NaCl solutions:

There was retardation in the growth of the seedlings incubated in the 1/4<sup>th</sup> and in the 1/8<sup>th</sup> dilutions of sea waters compared to their respective controls in Petri plate or soil (Fig. 2d and 2e). In 1 week old seedlings, the roots were longer than the respective shoots among all four cultivars in the controls as well as in salt stressed seedlings (Fig.3). In the control seedlings the roots are two times longer than the respective shoots (Fig.3A). In the salt stressed samples (1/4<sup>th</sup> dilution), the roots are 4 to 8 times longer than their respective shoots, the increase being 7/8 fold in the cvs IP 22269 and IP 6098 (Fig. 3B), indicating the stress to be more in these cultivars. In the 1/8<sup>th</sup> dilution of sea water, in one week old seedlings, the roots are longer than the respective shoots, increase being 2to 4 fold among the cultivars Fig. 3B). On the other hand, the proportion of root length to shoot length was at par with that of their counterparts in controls in the cvs IP 19612 and IP 6098, while it was different in the cvs IP 6106 and IP 22269 (Fig.3C). Control plants in the field grew healthy, (Fig. 2h) and the plants in the pots irrigated with salt waters were week but survived (Fig.2i).

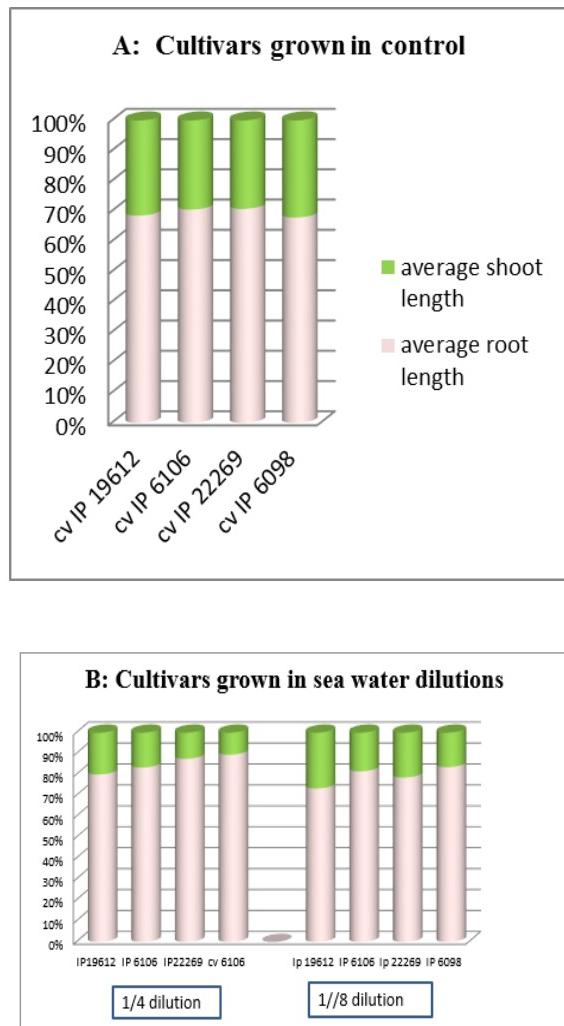
#### C. Response of cultivars IP 6106 and IP 22269 to MS+3.0 mg/L BAP culture media:

One week old control seedlings of cultivars IP 6106 and of IP 22269 each had two leaves and long roots at the time of culture in the MS+BAP medium (Fig.4 a). In four weeks of culture in MS+3mg/L BAP medium, each shoot developed 4-6 shoots (Fig. 4b). The multiple shoots could be separated into individual shoots easily. The single shoots subcultured in MS+1.mg/L NAA medium developed roots in two weeks (Fig. 4c and d). The 2 week-stressed seedlings (1 week in 1/4<sup>th</sup> dilution of sea water and 1 week against 150mM NaCl solution) of cultivars IP 6106 and IP 22269 were week compared to the respective controls (Fig. 4 j). The seedlings of cv IP 22269 cultured in MS+3.0mg/L BAP medium remained single shoot up to 4 weeks, which developed 3 multiple shoots after another four weeks (Table 1, Fig. 4f - h). The outermost leaves were necrotic and died (Fig. 4h). But new shoots formed which could be separated into single shoots, upon subculture in MS+ 1.mg/L NAA medium

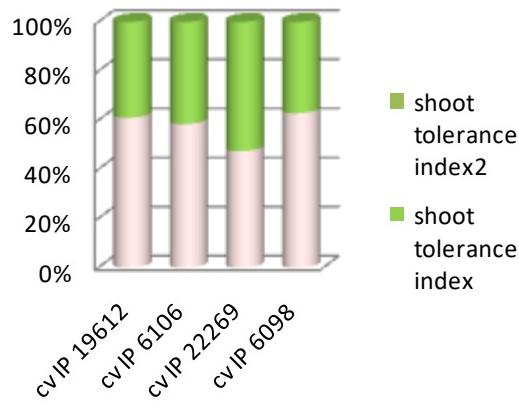
developed roots in 3-5 weeks. The seedlings of cv IP 6106 in BAP medium developed 2 shoots in 8 weeks (Fig.4i - l). The old leaves are necrotic and withered. The single shoots subcultured in MS+ 1.0 mg/L NAA medium rooted in about 5 weeks. The plants looked weaker than the plantlets of IP 22269. The plantlets of controls are hardened in the 1/4 diluted MS liquid medium for 1 week and could be transferred to autoclaved soil. The plantlets of salt stressed are being hardened in the 1/4<sup>th</sup> MS medium (Figs. 4e and m).

#### D. Response of cultivars IP 6106 and IP 22269 to MS+ 2.5 mg/L 2,4-D culture media:

The leaf explants of one week old control plants of cultivars cv IP 22269 cultured in MS+2.5mg/L 2,4-D medium callused in a week (Fig. 4 n and o). Callus initiated readily from the leaf base if the explant included the base or from injured region if the explant included it (Fig. 4 o, Table 1). The leaf explants of the stressed plants (150mM NaCl) in both the cultivars callus initiated from the coleoptile and also from the leaf explants in one -two weeks after inoculation, (Fig. 4 p). The callus from the stressed explants turned necrotic and only in a few explants it proliferated (Fig. 4p and Q). The differentiation of the calli is being carried out.



#### C: Cultivars grown in 150mM NaCl solutions



**Figure 3** Ratio of root: shoot length of 1 week seedlings

#### 4. DISCUSSION

##### A. Effect of salinity on emergence and seedling growth:

One of the predominant effects of salinity is delay in seedling emergence and decrease in per cent seed germination. In the present investigation, the seed emergence varied between 1 to 4 days in the salt solutions and sea water dilutions. All four genotypes germinated in 1 day in the NaCl salt solution, while in the diluted sea waters the seeds germinated by the 3<sup>rd</sup> or 4<sup>th</sup> day. Salt at 150mM concentration did not delay emergence. However, NaCl affected per cent of seed germination. Differences were also observed among the four cultivars in per cent of seed germination in the sea water dilutions, which could be attributed to the genotypic differences. The differences noticed in a genotype in all three salt solutions reflect the treatments (effects of salts) rather than the genotypic differences that varied among the cultivars IP 6106, IP 22269 and IP 6098 (Fig.1). The cv IP 6098 had 74% seed germination in 150mM NaCl solution, which was drastically reduced to 42% in 1/4 diluted sea waters with similar EC (of 11dS/m), suggests its sensitivity to other cations and anions present in the sea waters than NaCl. Decrease in per cent of seed germination with increasing salinity observed in the present study was reported in other varieties of pearl millet [7], *Hordeum*, *Brassica* [13] and in *Amaranthus* [14]. Similar effect of sea water salinity- the delayed emergence and fall in per cent of seed germination with increased salinity was also reported in three cultivars of tomato [15]. In the present study the seedlings were weak, with highly compromised shoot growth and elongated roots in the stressed conditions. The effect is more pronounced in the 1/4<sup>th</sup> diluted sea water than in the 1/8<sup>th</sup> diluted sea waters or NaCl solutions among all four cultivars. Salinity showed deleterious effect on the vegetative growth and root length of the seedlings under salt stressed conditions in many crops including varieties of pearl millet [7], sorghum [16], and tomatoes [15]. Although specific tests have not been carried, the disproportionate growth of root and weak seedlings under salt stress may be attributed to osmotic stress that has been reported in

musk-melon under salt stress [17] or due to ion toxicity observed in barley [13].

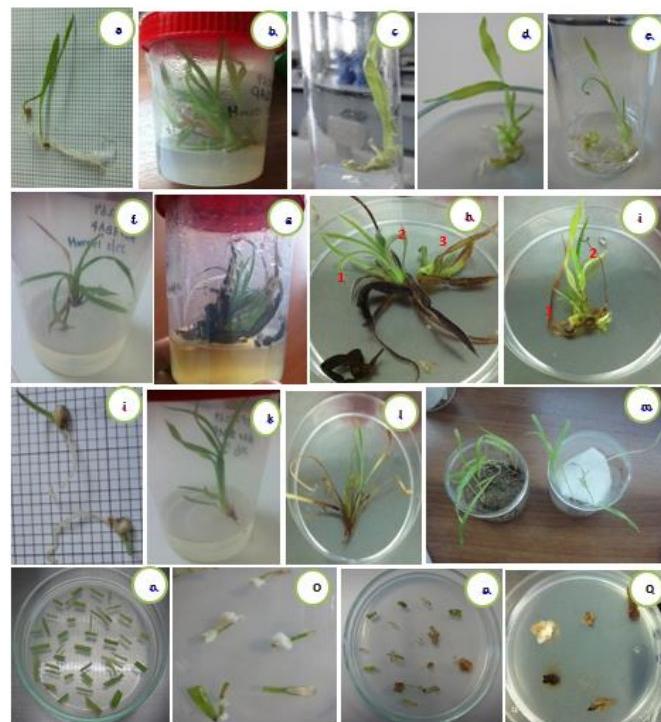
#### B. The response of the four cultivars to *in vitro* conditions:

The response of cvs IP 6106 and IP 22269 to BAP supplemented MS media are similar. The control seedlings were healthy at the time of culture; each single shoot yielded a maximum of 6 shoots in a month's time. The salt stressed seedlings of either cultivar were weak at the time of culture; developed into 2-3 multiple shoots in 8 weeks. The oldest leaves of the salt stressed seedlings died probably due to higher levels of ABA, which was reported in the salt stressed seedlings of barley [18]. Although there was delay in the multiple shoot development and lesser number of shoots per seedling, the response of the salt stressed seedlings to the BAP medium appears to be similar to that of controls. The salt stressed cultivars of pearl millet had higher amounts of proline and K<sup>+</sup> ions as a mechanism to tolerate salt stress [19]. If the present cultivars also accumulate proline and K<sup>+</sup> under stress, probably longer time is needed to ameliorate the effect and yield multiple shoots. BAP is reported to stimulate leaf growth in water deficit conditions in wheat [20], which suggests that higher concentrations of BAP may be required to obtain more number of shoots from the salt stressed seedlings. The extrapolation of the present result to the other salt tolerant cultivars will enhance the observed and speculated response. The response of leaf explants of the stressed and unstressed seedlings is similar to callusing (MS+2,4-D) medium. The explants of stressed seedlings if contained proline, the amount in the explant may be minute compared to the amount in the entire seedling and so the explants callused in one/ two weeks similar to those of control seedlings. Besides, the hormone 2,4-D could negate proline content in the explants, as it is reported to decrease proline content in the seedling growth of bamboo [21]. The differentiation results from the control seedlings and salt affected samples are awaited.

**Table1** Response of Control and salt stressed seedlings to MS+3.0mg/L BAP media and MS+2.5 mg/L 2,4-D media

Cultivar	Response of 1 week old seedlings to MS+3.0 mg/L BAP medium control stressed	Response of leaf explants to MS+2,4-D control stressed
IP 22269	Response in 4 weeks, 42% formed multiple shoots. Each seedling formed 4-6 shoots.	Response in 8 weeks. 20% formed multiple shoots, each formed 2-3 shoots.

IP 6106	Response in 4 weeks, 29% formed multiple shoots. Each formed 3-5 shoots.	Response in 8 weeks. 10% formed multiple shoots, each formed 2 shoots.	32% Callused in 2 weeks. Friable callus, proliferating.	6% callused in 2-3 weeks. Scant callus, slow proliferation.
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**Figure 4** *In vitro* response of cv IP 22269 and IP 6106 to culture media

Figure a: 1 week old control seedling of IP6106.

Figure b: Seedling in Fig. 1a with multiple shoots in 4 weeks in MS+3.BAP medium.

Figure c: Separated shoots of IP 6106 cultured in MS+1.0mg/L NAA medium.

Figure d: Single shoot of IP 6106 after root formation.

Figure e: plantlet in Fig.d in 1/4 MS medium for hardening.

Figure f: Seedling of cv IP 22269 stressed against 150mM salt solution for 2 weeks, cultured in MS+ 3.0mg/L BAP medium for a month.

Figure g: Seedling of Fig. f after 8 weeks, with multiple shoots, phenolics in the medium changed the color of the medium.

Figure h: Seedlings from Fig. g, with 3 multiple shoots, before subculture in MS+ 1 mg/L NAA medium. Oldest leaves dead.

Figure i: Seedling of cv IP 6106 each salt stressed seedling developed 2 multiple shoots in 8 weeks, before subcultured in MS+NAA medium.

Figure j: Slat stressed seedlings of IP 6106 at the time of culture.

Figure k: Seedling of Fig. j after 4 weeks in MS+ 3.0mg/L BAP medium.

- Figure l: IP 6106 seedlings with 2 multiple shoots, before subculture in MS+ 1 mg/L NAA medium.
- Figure m: Control plantlets of IP 6106 in 1/4 MS liquid medium and in autoclaved soil.
- Figure n: Leaf explants of control seedling IP 22269, cultured in MS+2.5mg/L 2,4-D medium.
- Figure o: Leaf explants of IP 22269 with white, friable callus in 2 weeks.
- Figure p: Leaf explants from stressed seedling cultured in MS+2.5mg/L 2,4-D medium.
- Figure Q: Stressed leaf explants of cv IP 22269 callused in 2 weeks, with white, friable callus.

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## ACKNOWLEDGMENT

The authors thank UMS for supporting this work; MY and NP thank UMS for providing all the facility.